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TENKA,Z.et al.

EXPERIMENTAL CONTRIBUTIONS TO THE LYMPHATIC PATHOGENESIS OF ANTHRAX INFECTION.(Transl.from Ceskoslovenska microbiologie, 1958, 3: No.2, p.82-91. Transl.by Claudius F.Mayer,M.D.,Washington,Sept.,1959).

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In reference to the sensitiveness toward anthrax infection by the different pathways of infection,it has been unanimously shown in all works that the most sensitive is the cutaneous entrance. For these reasons, BEZREDKA(1926) concluded that the skin is the organ of anthrax infection and of anthrax immunity. It is however known that the anthrax infection can be also provoked in other ways, especially by injury of the mucosal covers, by the alimentary way and by inhalation(HEUŠKA,1927; SOBERNHEIM,1931).

It was shown that,at the inhalation of the infection, the anthrax microbes do not remain in the lung tissue, but they pass from the lungs into the mediastinal lymph nodes(BARNES,1947; HENDERSON,PEACOCK & BELTON,1956; ROSS,1957). At the study of the cutaneous infection,we have demonstrated(TENKA et al., 1956) that the skin's sensitiveness depends upon the possibility of the microbes' penetration into the lymphatic vessels and that with the injection of the spores directly into the lymphatic nodes,we get a similar or even increased sensitiveness toward the infection. This actuality is in agreement with the known fact that each intracutaneous injection of any kind of matter has first of all a lymphatic resorption(HUDACK & McMASTER,1933; ROWSON & MORGAN,1955). Later, WIDDICOMBE et al.(1956) published a work on the lymphatic pathogenesis of anthrax.These authors have followed the quantitative cultivation of an amount of anthrax microbes in the lymph,taken before its entrance into the lymph nodes and at its passage through the lymph node, and in this way they ascertained the remarkable multiplication of these microbes.

The object of this work is then to deepen the knowledge about the importance of the lymphatic system in the pathogenesis of anthrax,and to elucidate the entire dynamics of the development of anthrax infection.We come from our experience to the statement that,briefly,by inoculation of the infection it can be assured that spores

are caught in the regional lymphatic nodes(TENKA et al., 1956). On the other hand, the work is based upon a few newer methods of physiopathological observation of the lymphatic system(MÁLEK & KOLC,1957) and upon the systematic study of the importance of the lymphatic system in the pathogenesis of infection and intoxication, for instance,in tetanus(MÁLEK,KOLC & ŽÁK,1957).

MATERIAL AND METHODS.

STRAIN.- In all series of experiments,we have been using suspensions of spores which we prepared from standard lyophilized stock of the spores of the U-5 strain of Bacillus anthracis. We have always diluted the suspension before use, and have counted the microbes by pouring the suspension on agar plates.

COUNTING OF SPORES AND BACTERIA.- The large number of spores or vegetative forms of bacteria both from the spore suspensions and from the separate tissues and organs (after preceding pulverization)(in small dishes) we have diluted with physiological (saline) solution,and from the individual dilutions we poured on 2-4 agar plates. On the 24-hour cultures we counted the number of outgrown colonies,and we always determined the resultant number from the average values of the culture plates in 2 to 3 calculatory dilutions. The number of the outgrown colonies from the various dilutions was never different at recounting.

(p.83) INFECTION OF SMALL ANIMALS.- We infected rabbits and guinea pigs subcutaneously and intradermally according to the current method,at the rear extremities. Into the popliteal nodes we infected by the following methods:- we exposed the node by a small incision so that it was denuded at its convexity,and we took care that we should not injure either the afferent or the efferent lymphatic vessels. We injected the required number of spores into the nodes,always as much as possible in the smallest amount of fluid(0.05 to 0.1 ml). Into the lymphatic vessels,we injected at the site between the para-aortic left node and the lumbal cistern. We gained access to the vessel by laparotomy in the midline. As control groups for comparison, we used rabbits which after the laparotomy we injected with a spore suspension into the spleen,and rabbits infected subcutaneously or at the skin. Different amount of spores was always injected into the experimental groups of animals in equal amount of fluid. We have infected sheep by intracutaneous puncture into the shaved part of the left hing extremity.

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PROPAGATION OF INFECTION.- We have followed up the infection in the rabbits and guinea pigs by cultivation at definite time intervals introduced in the experimental period. We killed the animals by bleeding them to death with the aid of a heart puncture; we took out organs, and,by pulverizing them in small dishes,we carried out the cultivation. We cultivated blood, urine, lymphatic fluid from the cistern by lumbal puncture(only in rabbits), inguinal and para-aortic lymph nodes, namely both from the infected and from the opposite side, furthermore spleen,liver,lungs,and the site of the injection.

For a permanent follow-up and observation of the presence of B.anthracis in the blood and lymph, we prepared sheep in the following way:- to sheep of about 30 Kg in weight each, before the operation we have given 100 mg of heparin intravenously. In a slight pentothal anesthesia, we exposed the thoracic duct by a section at the neck, and introduced into it a polyethylene tubule(cannula) to the length of about 1,5 to 2 cm. The other cannula was introduced into a branch of the left jugular vein and we connected both cannulae with a short rubber drain tube.Thus, we obtained an number of repeated taking of lymph while preserving the lymph-blood circulation for the entire duration of infection. We took blood by means of the polyethylene cannula introduced into a branch of the right jugular vein, to the lumen of the vessel. During the duration of the experiment,we administered 50 mg of heparin to the sheep at 4-hourly intervals so that we would prevent the obstruction of the cannula by a blood coagulum. The administration of heparin does not influence the course of anthrax infection,as we have made it sure with a parallel experiment on rabbits which we provided with adequate amount of heparin at the neck,and at similar intervals as the sheep.

RESULTS.

PROPAGATION OF ANTHRAX INFECTION IN RABBITS AND GUINEA PIGS.

We have infected a group of rabbits subcutaneously with 10 to 20 LD_{100} of spores in 1 ml. We have killed the rabbits with a heart puncture at different intervals from 8 to 144 hours as it is evident from TABLE 1. From the killed animals,we took material for cultivation. The results are summed up(TABLE 1):- from the killed animals in the period of time from 8 to 72 hours after the infection,we have obtained predominantly negative cultures from all materials. Yet, during this time section, for a great number of microbes we get frequent proof of their individual destruction in the lymphatic nodes. In two animals(for 45 and 74 hours) we found the pre-

sence of B.anthracis in the lymph, in two further rabbits(for 42 and 72 hours),otherwise with entirely negative findings, we have cultivated microbes from the lymph and the lungs.Further positive findings indicate that, beside the lymph and the lungs, the microbes could be caught in the spleen and the liver, while ,still in the same time, the cultivation from the blood was negative(rabbits killed after 45,70 and 120 hours). Only the later phase of the infection which is at individually different time intervals in a few animals, is accompanied by positive cultural finding in the blood and in all tissues of the animals(Tab.1).

Because already by 8 hours after the beginning of the infection we had not found bacteria present in the organs which could be cultivated we made also use of larger infectional doses(5 million spores) ,and we cultivated at short time intervals, namely at 5,10,15,and 30 minutes, and at 1,2,and 4 hours. In these short time intervals, we found spores not only in the subcutaneous tissue(at the site of the puncture) and in the regional lymphatic nodes, but also in the urine and isolatedly in the blood(Table 1). From this it is obvious that the subcutaneous injection of the spores leads,beside their localization in the lymphatic nodes, also to a direct invasion into the blood through the subcutaneous capillaries.

We divided into four groups the cultural results in rabbits which were acquired in the course of the anthrax infection:- the first group sums up the cultural results shortly after the dispersion of the spores from the site of puncture— to 4 hours after the beginning of the infection.The second group is chronologically attached to the first group and it lasts for a different length according to the individual course of infection in the individual animals. It is characterised predominantly by negative findings from the lymphatic nodes and organs.(p.84) The third group of findings again distinguishes itself individually chronologically according to the rate of the course of the infectious process. It is characterised by the evidence of the microbes at cultivation from the lymphatic nodes, from the lymph of the ductus thoracicus and by the rare finding from some internal organs,first of all from the lungs and then also from the spleen and the liver. The last group of findings is characterised by the appearance of the microbes in the blood. This is the result of a collapse of the defense of the organism, and shortly afterwards the animal perishes.

TABLE 1.

(see over)

TABLE 1.: SYNOPSIS OF CULTURAL FINDINGS IN RABBITS AND GUINEA PIGS KILLED AT VARIOUS TIME INTERVALS.

(HEADINGS of vertical columns: A) Number of animals killed before 4 hours after the infection; B):I.GROUP: positive cultural findings before four hours after the infection; MIX(lymph nodes, organs, blood urine); C) Number of animals killed from the 8th hour on after the infection; D):II.GROUP: Negative cultural findings from the 8th hour of the infection on; E) III.GROUP: positive cultural findings in the lymph, lymphatic nodes,--isolatedly also in other organs,with negative blood from the 8th hour of the infection on; F):IV.GROUP: positive findings in the blood and in all organs).

RABBITS: A) 14.- B) minutes after the infection: 5,5,10,15,15,30,30 min., 60, 60 and 120 min.- C) 49.- D) 16 animals-- hours of infection: 8,12,24,36,36,41,42,48, 48,73,73,85,87,117,120,and 121 hours.- E) 7 animals--hours after the infection: 42, 45,45,70, 72,74,120 hours;- F) 26 animals--- hours after the infection: 41,48,48, 48,51,51,55,60,63,66,72,72,72,72,72,79,80,96,96,96,96,96,96,120,120,and 144 hours.

GUINEA PIGS:_ A) 12.- B) minutes after the infection: 30,240 and 240 min.- C) 77.- D) 44 animals--hours after the infection: 7,7,7,8,8,8,11,11,11,12,12,15,15,15, 16,16,16,25,25,26,26,28,28,29,39,39,40,40,47,47,48,48,48,56,56,56,57,57,58,58,59,59, 73,hours; E) 11 animals-- hours after the infection: 12,25,28,39,56,58,59,67,67,73,73 hours.- F) 22 animals--hours after infections: 40,48,72,72,72,72,72,72,72,72,72,72 72,72,73,73,73,80,80,120,120,and 144 hours.

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We did a similar research on guinea pigs which we injected intradermally with 20 LD in 0,1 ml into the skin of the left lower part of the abdomen.This area has a lymphatic drainage to the inguinal nodes. From the first group,shortly after the infection(before 4 hours) we obtained positive findings from the site of the injection and in isolated cases from the corresponding lymph nodes. In difference from those rabbits which we have infected subcutaneously,in the guinea pigs after the intracutaneous infection in not a single case did we find dissemination of the spores by the blood into the organism. The second group(from the 4th hour of the infection on) was in harmony with the group of rabbits of similar time limit. This goes for the culturally negative findings both from the lymphatic nodes and from the organs.Only in a single case could we cultivate the B.anthracis from the regional lymph node 12 hours after the infection. In the third group are animals in none of which does the

multiplication of the microbes begin in the lymphatic nodes,and it comes to the first penetrations of the bacteria(p.85) with the lymph into the organism. This process begins by the 25th hour after the infection and it is individually different according to the rate of the course of the infection(See Table 1). In 8 guinea pigs, we found positive cultural findings only in the lymphatic nodes(after 39,56,58,59, 67,67,73,and 73 hours), and in two animals we found positive findings in the lymphatic nodes, in the lungs, and the spleen by 25 and 28 hours after the infection, at which the rest of the organs and the blood remained negative. This period is completely in agreement with the spread of the infection in the rabbits. Likewise the subsequent stage is also in harmony with the corresponding stage in the rabbits; it has an individual time run,and it is characterized by the appearance of the microbes in the blood,by which the lethal end of the infection is also dated in a few following hours.

FIG.1: INFLUENCE of the cells of the lymphatic nodes upon the growth of B.anthracis.In rotating test-tubes ,in the presence of 22 million lymph cells,isolated from lymph nodes,we have cultivated 100(Curve C), 1000(Curve B), and 10,000(Curve A)spores,and after 2,4,6,10,and 20 hours,we determined their number. The X axis:— the period of cultivation in hours; the Y axis:— the number of spores.

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The negative cultural phases could not be explained so that at a definite period there would not be any microbes present in the organism.It comes probably to it that in the cultivated organs the microorganisms are in a small number and during the preparations of the tissue homogenates and their pouring upon the plates, it comes to their destruction. In order to be able to confirm this supposition experimentally,we have cultivated,together with pulverized lymph nodes, 50 spores and we have repeatedly found that on every plate isolated colonies have grown only. For a more profound knowledge of the dynamics of the influence of the lymphatic tissue upon the spores,we have cultivated different quantitites of spores together with isolated lymph cells in vitro,and by quantitative cultivation we have followd up their growth(Fig.1). The curve with 100 spores has demonstrated to us that in the course of the first hours/the cultivation/comes to considerable decline of the cultivated it microbes,however the single surviving microbes multiply anew after a certain delay. Just by this fact alone, the culturally negative period can be explained; in the lymphatic nodes,isolated,culturally not intercentable microbes remain only,which

later will multiply in the organism.

SENSITIVENESS OF RABBITS TO ANTHRAX INFECTION AFTER INJECTION OF SPORES INTO THE LYMPHATIC SYSTEM.

The finding that the spores, injected into the animals intradermally and subcutaneously, will be localized at the regional lymphatic system, led us to the question whether we could experimentally confirm how the susceptibility of the organism to the infection is influenced directly into the lymphatic system. We carried out an experiment in three groups of rabbits. We injected the first group with various amounts of spores subcutaneously, into the spleen, into the lymph, and into the popliteal nodes. We have found that after the infection directly into the lymph and into the lymph nodes, more animals perished and in a shorter period of time than after a subcutaneous infection or after the infection into the spleen. (Table 2). The infection progressed in the same way in the second group (Table 3) where we have compared the subcutaneous infection and the infection into the popliteal nodes. The infection into the popliteal nodes had again not only a higher mortality but also the whole period of survival was shorter in contrast with the subcutaneous infection. The third group in which the subcutaneous infection was compared with the infection into the spleen, into the lympha and into the popliteal nodes, likewise indicates that the quickest is the course of infection after an injection into the lymphatic system (Table 4). From the presented experimental results it can be concluded: with spores inserted, without previous localization in the skin, or under the skin, directly into the lymphatic system, the infection quickens and it becomes lethal even at smaller number of spores.

(For Tables 3, 2, and 4 see next page).

DYNAMICS OF THE SPREAD OF INFECTION BY THE LYMPHATIC SYSTEM IN SHEEP WITH AN ARTIFICIALLY CREATED LYMPHO-VENOUS COMMUNICATION.

In order to enable ourselves to follow the sector of the development of the infection which is characterized by positive cultures from the lymphatic tissues and isolatedly from the organs, in the sheep we have surgically prepared a lympho-venous fistula where we have taken lymph at regular intervals.

In all experiments of animals we have cultivated the microbes from the lymph, to begin with. Only 10- to 16 hours after the first findings of microbes in the lymph did it come to a positive culture in the blood (Table 5). After the initial growth

TABLE 2: SUSCEPTIBILITY OF RABBITS TO VARIOUS WAYS OF INFECTION.

a) METHOD OF INFECTION	b) NUMBER OF SPORES	c) NUMBER OF VACCINATED PER NUMBER OF DEAD	d) SURVIVAL IN HOURS
subcutaneous	800	3/1	120
	8000	3/2	120,168
spleen	8000	3/2	96,144
	50000	3/3	96,120,144
lymph	80	3/2	44,79
	800	3/3	54,58
	8000	3/3	38,48,58
popliteal node left	80	3/2	70,81
	800	3/2	37,62
	8000	3/3	44,62,64

TABLE 3: SUSCEPTIBILITY OF RABBITS TO VARIOUS WAYS OF INFECTION

subcutaneous	400	5/0	66,66
	4000	5/2	42,42
popliteal nodes	400	5/2	
	4000	5/5	28,28,42,42,66

TABLE 4: SUSCEPTIBILITY OF RABBITS TO VARIOUS WAYS OF INFECTION.

intracutaneous	10	5/0	-
	50	5/1	91
	500	5/3	51,91,91
	5000	5/3	34,36,52
spleen	10	5/0	-
	50	5/0	-
	500	5/2	52,66
	5000	5/4	53,54,54,85
lymph	10	5/0	-
	50	5/1	54
	500	5/3	72,72,74
	5000	5/5	37,42,53,54,54
ponliteal node	10	5/0	-
	50	5/0	-
	500	5/4	29,51,66,66
	5000	5/5	26,27,28,35,51

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TABLE 5: CULTURAL results from lymph and blood in sheep with a created lympho-venous communication. (Vertical headlines: a) ordinal number of sheep; b) hours of infection; c) material from which culture has been made).-(Words in Rubric b),from top to bottom: 1) died at 59th hour; 2) died at 61st hour; 3) the lympho-venous cannula became obstructed at the 18th hour after the infection; died at the 54th hour; 4) died at the 64th hour; 5) died at the 52nd hour.- WORDS in rubric c): lymph ex blood(and so on alternately).

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FIG.2: NUMBER of bacteria in the lymph and the blood in sheep No.4 in the course of the infection. Dotted line - - - number of microbes in the lymph; —— full line: number of microbes in the blood. The end of the curve signifies the death of the sheep.The x axis: hours after infection: the Y axis: number of microbes in 1 ml.

FIG.3: NUMBER of microbes in the lymph and blood of No.5 sheep in the course of the infection. Dotted line- - - number of microbes in the lymph; full line—— number of microbes in the blood. The end of the graph means the death of the sheep. The X axis: hours after infection; the Y axis: the number of microbes in 1 ml.

(text.cont.)...the number of microbes was maintained in the lymph up to the lethal end of the infection substantially at euqal(equal) height.On the contrary, the number of microbes in the blood(appearing with deuly,in comparison with the lymph) has been continuously steeply increasing up to the time of death(Fig.2 and 3). By this experiment we corroborated that it comes to the spread of infection after the multiplication of the microbes in the regional lymph nodes, from where, with the lymph and with a swoop into the venous river bed,it arrives at the internal organs.

DISCUSSION.

It is known that the lymphatic system and most of all the lymphatic nodes are a very important filter for the microorganisms and for other corpuscular heterogenous particles(MANFREDI & VIOLA,1899; DRINKER,FIELD & WARD,1934; WIDDICOMBE,HUGHES & RAT,1955). Chiefly by immunization,this barrier capability of the lymphatic nodes is continuously raised,and it is considered as one of the chief defense reactions which protect against the spread of the infective microorganisms(BERMAN & SLAVAJSKA 1945). In contrast with this,we find,however, that the majority of the pathogenic microorganisms is first of all holding fast in the lymphatic system, where afterwards they multiply in the lymphatic nodes(lymphadenitis tuberculosa,brucellosa,tularemica,pestis,etc.) Such facts lead a few workers to the conclusion that the pathogenic microorganisms have become adapted to the exploitation of the metabolic process of the lymphatic tissues and they utilise it to their reproduction(PLANELES,1955).The two ideas seemingly contradictory to each other are however fully identical. They express the dynamic correlation of the defense of the organism and of the pathogenic aciton of the microbe. They show the importance of the lymphatic tissues for the pathogenesis and on the other hand the necessity of the modification of their metabolism in the course of the immunization process.

Our work presents a direct evidence for the lymphatic pathogenesis of anthrax infection on the ground of investigations of the dynamic infectious process,and it enables us to create for ourselves an idea about its course. Shortly, after the penetration of the spores into the tissues, it comes partly to their distribution along

the organism by the blood vessels, chiefly however, and this is important for the further development of the infection, to the settlement of the spores in the regional lymph nodes by the lymphatic pathways. The blood delivery of the infection, according to our findings, has no great importance for the pathogenesis itself, since in order to cause an infection with the insertion of spores directly into the blood stream, we have to use ten times larger doses than at the injection into the skin. As we are also showing in this work, the spores which have been localized in the lymphatic system, are partly killed by the inhibition from the tissues(possibly of peptid nature--CROMARTIE, WATSON & BLOOM, 1947). After adding the spores to tissue homogenates in vitro, and after an instantaneous cultivation it comes to a lowering of the number of the cultivated microorganisms. It is possible that we do not get hold of isolated vegetative forms or spores of B. anthracis which have settled in the lymphatic system, and thus we get periods which are culturally negative. The isolated microorganisms which are preserved in the lymphatic system begin to multiply with delay, but by the simultaneous production of toxic substances which attenuate the bactoricide capabilities of the organism(STERZL, TRNKA & LANG, 1956). The simultaneous creation of toxic substances has been experimentally supported by HARRIS-SMITH and others(1957) who succeeded already after a 5-hour cultivation in vitro to find the production of the toxin of B. anthracis. This toxic matter has undoubtedly a decisive influence upon the defense of the organism in the sense of a further reproduction and penetration of the reproduced microbes through the lymphatic barrier. The multiplication of the microbes is manifested by the first positive culture of the sample from the lymph. In this period we got hold of microbes in rabbits and in guinea pigs and both quantitatively and temporally at the lympho-venous fistula in the sheep. The microorganisms which, after getting loose, have arrived from the nodes with the lymph to the venous stream, were fished out from the blood by the bearest tissues as filters-- such as the lungs; therefore, in our material, there are cultural findings which repeate themselves a few times simultaneously in the lymph and in the lungs. For the further excess of microorganisms, washed out by the lymph into the blood, there are also set up other, very highly effective filtering organs-- the spleen and the liver. In this time the blood was culturally negative. The number of microbes in the lymph has remained all the time essentially equal, and this is pointing to a running leap of the microbes multiplying themselves at the periphery of the blood.

In such a period, it comes already both to a number of microbes even in the organs in which they get holded in the lungs, the spleen and the liver, and to a production of a large amount of toxin which directly effects both the defensive capacity of the leukocytes and their cleaning effect upon the microbes AND the cleaning factors of the blood serum(STEIZL, TENKA, & LANG 1956). Although it looks that the last arising of bacteriemia in the pre-mortal period is the consequence of a powerful multiplication and spread of the microbes just in these secondarily infected internal organs, the possibility is still unexplained whether it comes also to direct washing out of the microbes from the nodes into the venous stream(WIDDICOMBE et al., 1956); to this problem further attention must be still paid in the future.

An indirect proff that the death proper is not caused by the quantum of microbes but probably by a shock mechanism(SMITH et al., 1955) are the findings that bacteriemia of the animals dying from an anthrax infection is not always of such an extent that the number of the microbes could cause injury to the organs or biochemical changes or an anoxia of the tissues as it has been supposed earlier(CROMARTIE et al., 1946; KEPPIE et al., 1955; TENKA et al. 1956).

SUMMARY.

1. We have investigated the spread of the anthrax infection by means of quantitative cultures from the site of the injection, from lymphatic nodes, from the blood, and from the organs after inoculation of the spores subcutaneously and intradermally. Already shortly after the inoculation(after 4 hours) the cultural findings have been negative. In the further phase of development of the infection, however, we have caught microbes in the regional nodes, in the lymph, and in case of larger spread of the infection, already even in the lungs and in the spleen. Only in the last stage of the infection does it come to the appearance of the microbes in the blood and with that to a positive culture from all tissues of the body.

2. We have found that after the injection of various doses of spores directly into the lymph nodes, perishes, in comparison with the subcutaneous infection, a larger amount of animals at lesser dose and in a shorter time. The infection into the lymphatic tissues and intradermally has an almost equal effect.

3. The dynamics of the spread of the microbes by the lymphatic system has been currently found after the creation of a permanent lymho-venous connection in the sheep. We have shown in all investigated animals the presence of microbes in the lymph

about 10-16 hours earlier than in the blood.

The findings indicate the importance of the lymphatic tissue for the development of infection, and they are the foundation for the creation of the concept of the pathogenesis of anthrax.

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(THE RUSSIAN AND ENGLISH SUMMARIES ARE OMITTED HERE).

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END.